PORT DOCUMENTATION PAGE

1a

2a

2b. ...ICATION/DOWNGRADING SCHEDULE

1b. RESTRICTIVE MARKINGS

3. DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; distribution is unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
NMRI 90-37

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION
Naval Medical Research

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION
Naval Medical Command

6c. ADDRESS (City, State, and ZIP Code)
Bethesda, Maryland 20814-5055

7b. ADDRESS (City, State, and ZIP Code)
Department of the Navy
Washington, D.C. 20372-5120

8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research and Development Command

8b. OFFICE SYMBOL (If applicable)

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)
Bethesda, Maryland 20814-5055

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO
62233N	MM33C30.005	1051	DN249507

11. TITLE (Include Security Classification)
RECIPROCAL EXPRESSION OF HUMAN ETS1 AND ETS2 GENES DURING T-CELL ACTIVATION: REGULATORY ROLE FOR THE PROTOONCOGENE ETS1

12. PERSONAL AUTHOR(S) Bhat NK, Thompson CB, Lindsten T, June CH, Fujiwara S, Koizumi S, Fisher RJ, Papas TS

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM _____ TO _____	1990	5

16. SUPPLEMENTARY NOTATION
Reprinted from: Proceedings of the National Academy of Sciences, Vol.87, No.10, pp.3723-3727, (May 1990)

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
c-ets gene family
cell proliferation
gene regulation

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL
Phyllis Blum, Information Services Division

22b. TELEPHONE (Include Area Code)
202-295-2188

22c. OFFICE SYMBOL
ISD/ADMIN/NMRI

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3723–3727, May 1990
Cell Biology

Reciprocal expression of human *ETS1* and *ETS2* genes during T-cell activation: Regulatory role for the protooncogene *ETS1*

(c-*ets* gene family/cell proliferation/gene regulation)

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Communicated by Robert C. Gallo, March 5, 1990 (received for review January 5, 1990)

ABSTRACT **The expression of the protooncogenes *ETS1* and *ETS2* has been studied in purified human T cells activated either by cross-linking of the T-cell receptor–CD3 complex on their cell surface or by direct stimulation with phorbol esters and ionomycin. Our results show that resting T cells express high levels of ETS1 mRNA and protein, while expression of ETS2 is undetectable. Upon T-cell activation, ETS2 mRNA and proteins are induced, while *ETS1* gene expression decreases to very low levels. Late after stimulation, ETS1 mRNA is reinduced and maintained at a high level, while *ETS2* gene expression decreases to undetectable levels. Therefore, it appears that in human T cells, *ETS2* gene products are associated with cellular activation and proliferation, while *ETS1* gene products are preferentially expressed in a quiescent state.**

The cellular c-*ets* family of genes have been identified by their sequence similarities with the viral v-*ets* oncogene of the E26 avian leukemia virus (1, 2). The c-*ets-1* (3–5), c-*ets-2* (3, 4), c-*erg* (6, 7), c-*elk-1*, and c-*elk-2* genes (8) are members of the c-*ets* family of genes. In humans, *ETS1* and *ETS2* are localized on different chromosomes (9) and code for distinct sets of mRNA (3, 5) and proteins (10–12). *ETS1* and *ETS2* code for structurally related proteins (70% similarity) (4) that are primarily localized in the nucleus (10, 13) and are capable of binding to DNA (13). The DNA-binding domain has been localized to the carboxyl terminus of ETS1 (14), a region highly conserved among all ETS family members (4). Both ETS1 and ETS2 proteins are labile nuclear phosphoproteins (10, 12, 15). They share many properties with other nuclear oncogene products like JUN, FOS, and MYC: nuclear localization, rapid turnover, and quick response to second messengers, suggesting that the c-*ets* gene products may also play a vital role during cell growth. In fact, the gene transfection and subsequent overexpression of murine *Ets-2* has recently been shown to abolish the serum requirement for cell growth and stimulate the proliferation of NIH 3T3 fibroblasts (16).

Although c-ets-1 mRNA is detectable in different murine (17–19) and human tissues (20, 21), c-ets-1 mRNA and proteins are expressed at high levels in the thymus. Thymus is the major site for T-cell development, differentiation, and functional maturation. Initially, the thymus is populated by $CD4^-$ $CD8^-$ (DN, double negative) cells, some of which will differentiate into $CD4^+$ $CD8^+$ (DP, double positive) cells. The single-positive $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ thymocytes emerge later and subsequently migrate from the thymus into peripheral lymphoid sites (22). We have shown that: (*i*) the murine *Ets-2* expression appears 1 day earlier than *Ets-1* expression, corresponding to both DN and DP blast thymocytes; (*ii*) the *Ets-1* expression begins in 18-day-old fetal thymocytes, coinciding with the appearance of single-positive thymocytes; (*iii*) both *Ets-1* and *Ets-2* genes are selectively expressed in $CD4^+$ $CD8^-$ thymocytes rather than in DN or $CD8^+$ $CD4^-$ subsets; (*iv*) both *Ets-1* and *Ets-2* expression are detectable in peripheral T cells (19).

T cells are very important components and regulators of the immune response. T cells are activated to proliferate by interaction between T-cell receptor (TCR) and antigen in conjunction with a major histocompatibility complex protein recognized on antigen-presenting cells (23). This interaction initiates a cascade of intracellular biochemical events leading to elevation of intracellular Ca^{2+} ions and activation of protein kinase C (PKC) (23). T cells can also be activated *in vitro* after direct activation of PKC by phorbol esters and increase of intracellular Ca^{2+} ions by ionomycin (24).

To understand the role of c-*ets* gene products in T cells, we have studied the expression and regulation of *ETS1* and *ETS2* genes during human T-cell activation and proliferation. In this paper, we report that the resting human T lymphocytes express high levels of ETS1 and very low levels of ETS2 mRNA and proteins. Upon T-cell activation, *ETS2* gene products are induced while the ETS1 mRNA and proteins are decreased. While *ETS2* gene products appear to be required for cellular activation and proliferation, the *ETS1* gene products may be involved in maintaining T cells in a quiescent state. Taken together, our results suggest that *ETS1* and *ETS2* genes may have a reciprocal relationship in the regulation of human T-cell activation.

MATERIALS AND METHODS

Cells. Resting human peripheral T cells were isolated from buffy coats obtained by leukophoresis of healthy donors as described (25). These cells were coated with saturating amounts of murine monoclonal antibodies (mAbs) 60.1 (anti-CD11), IF5 (anti-CD20), FC2 (anti-CD16), and 63D3 (anti-CD14). This mixture of mAbs coated all B cells, monocytes, large granular lymphocytes, and CD11-bearing T cells. The cells were washed three times and mAb-bound cells were removed by using goat anti-mouse immunoglobulin-coated magnetic particles. Cells obtained are >99% viable and >99% $CD2^+$ as determined by flow cytometry.

RNA Blot Analyses. Purified resting T cells were cultured in standard medium [RPMI 1640 containing 10% (vol/vol) fetal calf serum] with phorbol 12-myristate 13-acetate (PMA; 3 ng/ml) or phorbol 12,13-dibutyrate (3 ng/ml) or ionomycin (800 ng/ml) or a combination of PMA and ionomycin. An optimal amount of anti-CD3 antibody attached to a plastic surface was used to cross-link the TCR–CD3 complex as described (26). Cells were harvested, and RNA was isolated by the guanidinium isothiocyanate/cesium chloride method

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Abbreviations: TCR, T-cell receptor; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; mAb, monoclonal antibody.

(27). Total RNA in each sample was normalized with respect to 28S ribosomal RNA as described (26). Equal amounts of RNA were size-fractionated on a denaturing formaldehyde gel and blotted onto membrane. Probe preparation, hybridization, washing, and autoradiography were carried out as described (26). The size of ETS1 and ETS2 transcripts was measured by comparing the mobility of 28S and 18S rRNA and other RNA of known size detected on the blot. Consistently, a 6.8-kilobase (kb) major ETS1 RNA and three ETS2 mRNAs (4.7, 3.2, and 2.7 kb) were detected as reported (3). The 2.7-kb ETS2 mRNA could be detected easily from poly(A)$^+$ RNA blots as a faint band just below the 3.2-kb mRNA from total RNA blots.

DNA Probes. Human *ETS1* (PRD700)- and *ETS2* (CDNA14)-specific DNA fragments (3) were labeled with ^{32}P by nick-translation and used as probes. A 3′ end of a human *ETS1*-specific cDNA probe or an *ETS2*-specific probe H33 were also used in some experiments, which gave an identical pattern to those two probes described above (data not shown).

Immunoprecipitation and Fluorography. Resting human T cells were incubated with either standard medium (RPMI 1640 containing 10% fetal calf serum) or activated by the addition of PMA and ionomycin for 4.5 hr. Cells were resuspended in standard methionine-free medium in the presence or absence of PMA/ionomycin for 20 min. Cells were metabolically labeled for 60 min by using [^{35}S]methionine (Amersham; 300 μCi/ml; 1 μCi = 37 kBq), lysed in 2% sodium dodecyl sulfate (SDS)/50 mM Tris·HCl, pH 8.0/10 μg of aprotinin per ml, boiled for 5 min, and adjusted to radioimmunoprecipitation assay conditions as described (10). Insolubles were removed by centrifugation at 200,000 × *g* for 30 min. Lysate from the same number of cells was used for immunoprecipitation. Activated T cells incorporated 3–10 times higher amounts of [^{35}S]methionine into the trichloroacetic acid-insoluble proteins than did resting cells. The supernatant fluid was immunoprecipitated with either human ETS1- or ETS2-specific mAb (refs. 15, 28, and 29; S.K., unpublished data). The proteins were size-fractionated on denaturing polyacrylamide gel and processed for fluorography by using Enlightening (NEN) as described before (10).

RESULTS

***ETS1* Gene Is Expressed at a High Level in Resting Human T Cells.** We have shown in murine cells (19) that the levels of both *Ets-1* and *Ets-2* mRNA are lower in peripheral T cells than in thymocytes. The lower expression could be due to differences in either the microenvironment or the growth status of these cells. To test if c-*ets* genes are expressed at higher levels in quiescent vs. activated cells, we studied c-*ets* gene expression during T-cell activation and proliferation. Previously it has been shown that human peripheral T cells, when prepared as described in methods, are >98% pure and are in a quiescent state (25, 26, 30). The purified T cells used in this study are depleted of accessory cells and do not proliferate *in vitro* after stimulation with phytohemagglutinin, PMA, or ionomycin alone (26, 30). However, these T cells can be stimulated to divide by cross-linking the TCR–CD3 complex with a mAb attached to a plastic surface or by using the optimal amount of PMA and ionomycin as described (26). Under these conditions, >90% of the resting T cells are activated and the majority of the cells synchronously proceed through one round of cell proliferation. Total RNA was isolated from resting T cells after different time intervals following cross-linking of the TCR–CD3 complex. An RNA blot was prepared by loading equal amounts of RNA in each lane, as shown by ethidium bromide staining of the 28S rRNA band (Fig. 1 *Top*) and by the expression pattern of HLA genes (Fig. 1 *Bottom*). Resting human T cells expressed high levels of ETS1 and virtually undetectable levels of ETS2 mRNA (Fig. 1 *Middle*). Upon T-cell stimulation, ETS1

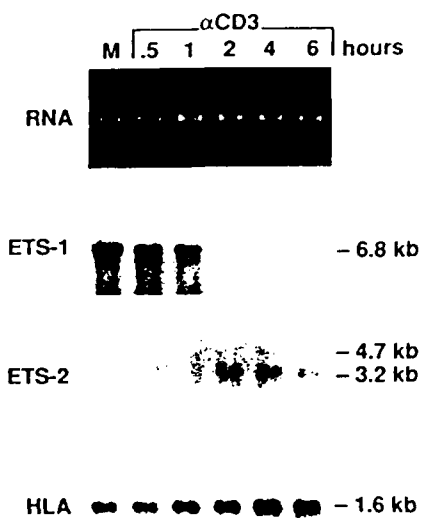

FIG. 1. ETS1 and ETS2 mRNA levels after cross-linking of the TCR–CD3 complex: Resting T cells were stimulated by cross-linking the TCR–CD3 complex with CD3-specific mAb G19-4. Total RNA was isolated from the cells prior to stimulation and after 0.5, 1, 2, 4, and 6 hr of activation. The RNA blot was prepared by using equal amounts of RNA and was probed sequentially with ETS1, ETS2, and HLA (HLA-B7)-specific cDNA probes. The transcripts detected by the probes are shown on the left. (*Top*) Ethidium bromide staining of the equalized 28S rRNA samples. Lane M, T cells after 6 hr incubation with medium alone.

mRNA decreased to undetectable amounts by 2 hr and remained at that level up to 12 hr (data not shown). By contrast, the ETS2 mRNA was induced within 2 hr, peaked around 4–6 hr, and remained at higher levels for up to 12 hr (data not shown). Therefore, we can conclude that the ETS1 mRNA is expressed preferentially in quiescent peripheral human T cells and decreases upon cell activation. On the other hand, ETS2 mRNA is induced after T-cell activation, an observation that is consistent with the findings that the murine *Ets-2* gene products accumulate during fibroblast and hepatocyte cell proliferation (17, 18).

ETS2 mRNA Can Be Induced in Resting T Cells by the Combination of Phorbol Esters and Calcium Ionophore. The binding of antibodies to the TCR–CD3 complex elevates intracellular Ca^{2+} ions and activates PKC (23). Both of these signal pathways are thought to be necessary for the activation and proliferation of T cells. PKC can be activated by phorbol esters, and Ca^{2+} concentration can be elevated by ionomycin. Addition of phorbol ester alone to resting T cells primes them to become blasts by 12 hr and maintains them in that state up to 24 hr. Stimulation of these cells with ionomycin results in their entry into the cell cycle. To understand how each of these two pathways contributes to the regulation of *ETS* gene expression after T-cell activation, we studied the expression of *ETS1* and *ETS2* genes in the presence of either phorbol 12,13-dibutyrate or ionomycin or both together. Addition of ionomycin or phorbol 12,13-dibutyrate alone had little effect on ETS2 mRNA level; however, when these reagents were added together, the level of ETS2 mRNA peaked at 4–6 hr, remaining at that level up to 24 hr. By contrast, the ETS1 mRNA level decreased up to 12 hr in the presence of either phorbol 12,13-dibutyrate or ionomycin; by 24 hr their levels were comparable to those of the resting T cells (Fig. 2). When added together, these two agents acted synergistically, causing an even more profound decrease in *ETS1* expression (Fig. 2).

To induce a more sustained activation of PKC, T cells were also stimulated with an optimum dose of PMA and ionomycin to promote maximal T-cell proliferation. When T cells are

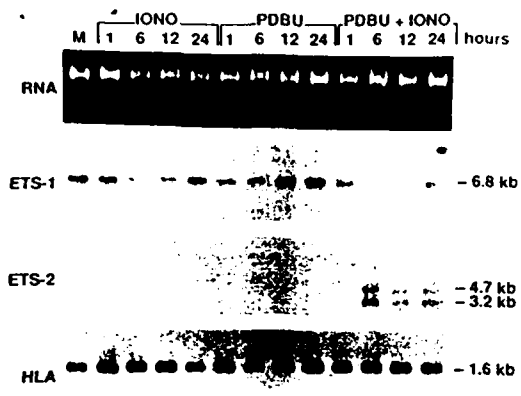

FIG. 2. Reciprocal relationship between *ETS1* and *ETS2* gene expression after stimulation of resting T cells with phorbol 12,13-dibutyrate (PDBU) and ionomycin (IONO). Purified quiescent T cells were stimulated with IONO, PDBU, or Iono and PDBU for the indicated time. Equal amounts of RNA were used to prepare blots. (*Top*) Ethidium bromide staining of the equalized RNA samples. (*Middle* and *Bottom*) Blots were sequentially probed with ETS-1, ETS-2, and HLA-B7-specific cDNA probes. Transcripts detected by the probes are shown on the left. Lane M, T cells after 24 hr of incubation with medium alone.

cultured in the presence of both of these agents for 3 days, ETS1 mRNA was reinduced to the levels observed in resting T cells. By contrast, the ETS2 mRNA level became undetectable at tnat time (data not shown). PMA and ionomycin added together had synergistic effects on both *ETS1* and *ETS2* gene expression, comparable to that effected by cross-linking the TCR–CD3 complex (compare Figs. 1 and 2). The level of HLA class I mRNA remained relatively unchanged during the drug treatment as reported (26). These results strongly suggest that both PKC activation and increase of Ca^{2+} ions play functional roles in *ETS2* induction and *ETS1* repression.

ETS1 and ETS2 Protein Levels in Resting Versus Activated T Cells. To determine whether the differences observed in the ETS mRNA levels are reflected at the protein level, the ETS1 and ETS2 proteins were examined by using well-characterized human ETS1- and ETS2-specific mAbs (refs. 28 and 29; S.K., unpublished data). In resting human T cells, all ETS1 proteins (p51, p48, p42, and p39 of 51, 48, 42, and 39 kDa, respectively) were expressed (Fig. 3). The lower levels of p42 and p39 detected in these cells could be the result of a high ratio of unspliced-to-alternatively-spliced ETS1 mRNA in quiescent T cells (C. Jorcyk, personal communication). These ETS1 proteins were undetected in activated cells (Fig. 3, compare lanes 2 and 4). In contrast, the ETS2 proteins are expressed at a high level in activated T cells and at undetectable levels in resting T cells (Fig. 3, compare lanes 1 and 3). Thus, we can conclude that during T-cell activation, the differences observed at the ETS1 and FTS2 mRNA levels are also reflected at the ETS1 and ETS2 protein levels.

Induction of *ETS2* Gene Expression by Phorbol 12,13-Dibutyrate and Ionomycin Requires New Protein Synthesis. To determine if new protein synthesis is required for the induction of *ETS2* and repression of *ETS1* gene expression during T-cell activation, resting T cells either were cultured with medium alone or were stimulated with phorbol 12,13-dibutyrate and ionomycin in the presence or absence of cycloheximide (an inhibitor of protein synthesis) for 2 hr. In resting T cells, ETS1 and ETS2 mRNAs were neither superinduced nor stabilized in the presence of cycloheximide (Fig. 4 *Middle*, lanes 1 and 2). However, during T-cell activation, new protein synthesis appeared to be required for ETS2 mRNA induction and to effect a decrease in ETS1 mRNA levels (Fig. 4 *Middle*, lanes 3 and 4).

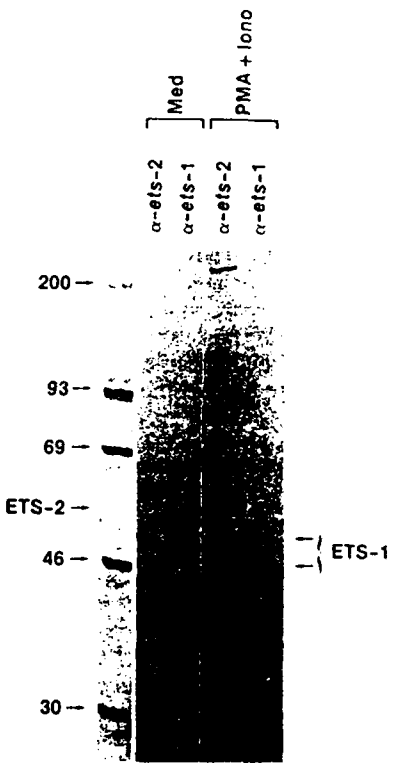

FIG. 3. ETS1 and ETS2 protein levels in resting vs. activated T cells. Resting T cells were cultured with medium (Med) or activated by addition of PMA and ionomycin (Iono). Cells were metabolically labeled with [^{35}S]methionine, and the cell lysate was processed for immunoprecipitation and fluorography as described. The ETS1 and ETS2 proteins are indicated by arrows. Isoforms of ETS1 proteins (p51, p48, p42, p39) are indicated by dots. The sizes (kDa) of the standard protein markers are shown on the left. α-ets-1 and -2, anti-ets-1 and -2 antibodies.

DISCUSSION

T-cell activation, proliferation, and its effector functions are regulated by binding of extracellular ligands (antigens, mito-

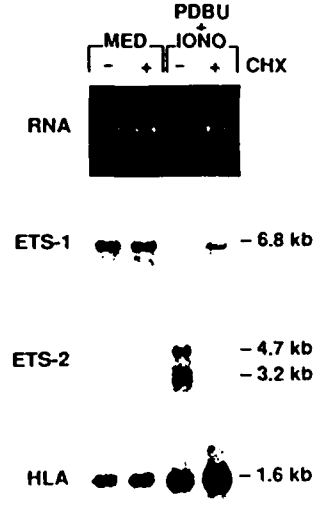

FIG. 4. Effect of the protein synthesis inhibitor cycloheximide (CHX) on *ETS* gene expression during T-cell activation. Purified resting T cells were treated with either medium alone or phorbol 12,13-dibutyrate (PDBU) and ionomycin (IONO) in the presence or absence of cycloheximide for 2 hr. RNA blots were processed as described in the legend to Fig. 2. Transcripts detected by the probes are shown on the left.

gens, and lymphokines) to cell surface receptors. Such interaction generates a cascade of intracellular biochemical events leading to activation of PKC, elevation of intracellular Ca^{2+} ions, and other second messengers (23). Generation of such signals results in the activation of resting cells, allowing them to emerge from G_0, and progress into the G_1 phase of the cell cycle. During cell proliferation, genes expressed in the G_1 phase of the cell cycle are positively regulated, while those genes expressed in the G_0 phase may be negatively regulated (31). Thus, the commitment of cells to proliferate depends upon how these two sets of genetic programs are regulated by second messengers that are stimulated by ligand–receptor interactions occurring at the cell surface level. During T-cell proliferation, both T cell-specific gene products such as lymphokines and a number of other gene products common to different cell types, including protooncogene products, have been shown to be expressed transiently (refs. 26, 30, and 32–35; for a review, see ref. 32). All of these genes can be grouped and categorized into immediate-, early-, and late-response genes, depending on their pattern of expression before or after cell division and their requirement for new protein synthesis during their induction (32). Both immediate- and early-response genes are induced prior to cell division. Expression of immediate-response genes is independent of new protein synthesis; for the induction of early-response genes, active protein synthesis is necessary. Late-response genes are expressed after cell division and also require active protein synthesis.

Results presented in this paper show that ETS2 mRNA and proteins are induced during the early phase of T-cell proliferation, thus enabling us to place c-*ets-2* genes along with other "early response genes" such as those encoding ornithine decarboxylase, *cyclin*, *myb*, and lymphokine gene products (32). In contrast, our results concerning *ETS1* expression in mature T cells demonstrate several interesting and unique properties: (*i*) *ETS1* gene is expressed at a high level in resting T cells and at an undetectable level in activated T cells; (*ii*) *ETS1* is reciprocally expressed when compared to the highly similar *ETS2* gene, which has been previously shown to abolish the serum requirement for resting fibroblasts and to stimulate directly cell proliferation (16). Collectively, these results suggest that *ETS1* gene products may be necessary to maintain T cells in a quiescent state. At this time it is difficult to assess if the *ETS1* gene products, alone or in association with other gene products, can accomplish this growth-regulatory task by either positively (as inducers) or negatively (as repressors) controlling those genes that are expressed in quiescent and activated T cells, respectively. In other cell types (non-T-cells), several gene products—e.g., statin (36), stress-inducible p188 protein (37), mammary-derived growth inhibitor (MDGI)-related 70-kDa protein (38), and six growth-arrest-specific genes (39)—also have been shown to be expressed at higher levels in quiescent cells but not in proliferating cells. In contrast to other genes, the protooncogene *ETS1* encodes a labile nuclear phosphoprotein with DNA-binding properties (10, 13, 14), which suggests that this protein may be a regulator rather than a marker of the quiescent state in human T cells.

Sequence analyses of mammalian c-*ets-1* and c-*ets-2* genes indicate that the carboxyl-terminal domain is highly conserved among many members of this family of genes (4). This domain contains the consensus nuclear localization signal starting at position 376: Gly-Lys-Arg-Lys-Asn-Lys-Pro-Lys (numbering as in ref. 4; for review, see ref. 40), a DNA-binding domain (14), and a putative consensus site [Arg-Xaa-Xaa-(Ser or Thr)] for the multifunctional Ca^{2+}/calmodulin-dependent protein kinase (41). We and others have shown that both c-ets-1 and c-ets-2 proteins are hyperphosphorylated upon activation of mouse thymocytes (12) or after cross-linking of the TCR–CD3 complex on human Jurkat T cells (refs. 29 and 42; S.K., unpublished data). This hyperphosphorylation occurs very quickly (within a few minutes) and appears to be due mainly to Ca^{2+}-mediated events (12, 29, 42). We have previously shown that the human ETS2

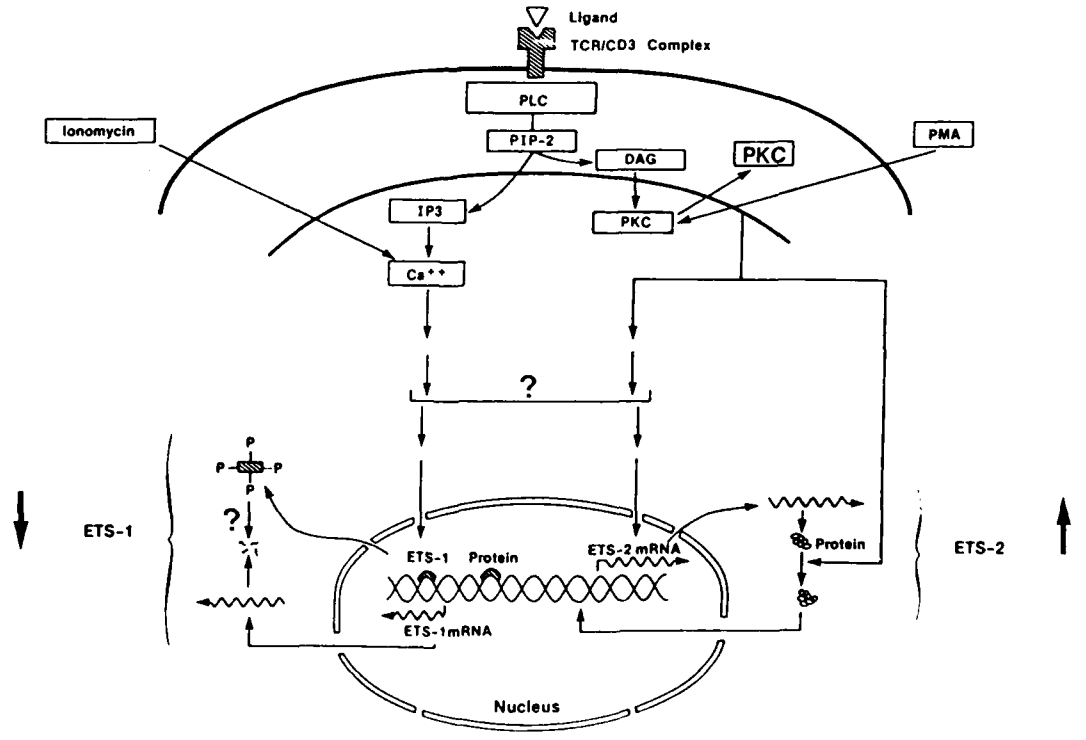

FIG. 5. Regulation of *ETS* gene expression during T-cell activation. The possible involvement of PKC and Ca^{2+} ions in stabilizing the *ETS2* and degrading the *ETS1* gene products is shown by the thick arrow pointing upwards and downwards, respectively. Thin arrows represent multistep mechanisms involved in the regulation of *ETS* genes. and . *ETS1* and *ETS2* proteins, respectively. The question mark signifies the pathways that are not completely understood.

protein has rapid turnover with a half-life of 20 min but is stabilized in the presence of activators of PKC (half-life ≥ 2.5 hr) (15). The hyperphosphorylated murine Ets-1 has lower affinity for DNA than the nonphosphorylated form (13).

Data presented in this paper along with other known properties of ETS1 and ETS2 proteins (10–15, 29, 42) are consistent with the model shown in Fig. 5. Stimulation of T cells by antigens, mitogens, or cross-linking of the TCR–CD3 complex results in the activation of phospholipase C and protein tyrosine kinase(s), which in turn results in the activation of PKC and increase of intracellular Ca^{2+} ions. Our results show that both of these signals are necessary for ETS2 mRNA induction and ETS1 mRNA degradation. At present, we do not know either the mechanism by which these second messengers regulate *ETS* gene expression or the nature of other gene products involved in this process. According to the model, during T-cell activation, *ETS1* gene products are rapidly lost from the cell not only by decreasing the level of ETS1 mRNA but also by rapidly phosphorylating the ETS1 protein. Positive regulation of the *ETS2* gene is achieved by induction of its mRNA and stabilization of the translated protein (see Figs. 1–3; ref. 15). We do not know if this reciprocal regulation of *ETS1* and *ETS2* gene expression is mediated by ETS1 or some other gene products; we have demonstrated, however, that new protein synthesis is necessary for this event. Thus, signals generated during T-cell activation have pleiotropic and reciprocal effects on *ETS1* and *ETS2* gene expression, the immediate effect being at the posttranslation level and a later effect being at the transcriptional or posttranscriptional level.

We have shown previously that the murine Ets-2 mRNA is induced during the early phase of hepatic (17) and fibroblast cell growth (18) and that this induction is independent of new protein synthesis (17, 18). However, in human T cells, *ETS2* induction requires new protein synthesis (Fig. 4). The *ETS2* expression follows a pattern similar (with respect to its induction and requirements for new protein synthesis) to that observed for *MYB*, another oncogene transduced by E26 virus (34). Since *MYB* gene products are shown to be required for the G_1–S transition (43), we can speculate that the *ETS2* gene products may similarly cooperate in this cell cycle transition. One can also speculate that the *ETS1* gene products, by themselves or in cooperation with other gene products, maintain T cells in a quiescent state by repressing immediate- or early-response genes or by inducing genes that are expressed at higher levels in resting state. Change in *ETS1* and *ETS2* expressions that we have observed during T-cell activation/proliferation could be due also to the results of other events that are more intimately linked with the proliferative state of the cell.

We thank Dr. D. K. Watson for different ETS1- and ETS2-specific probes, Mr. John Strouboulis and Ms. Rita Patel for their excellent technical assistance, and Ms. Susan Toms for typing this manuscript. We thank Dr. R. Ascione for his critical reading of the manuscript. This project has been funded at least in part with funds from the U.S. Department of Health and Human Services under contract number NO1-CO-74102.

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